



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12P 21/00, 21/02, 19/34 C12N 15/00, 15/10, C07K 7/00 C07H 15/12		A1	(11) International Publication Number: WO 91/05058 (43) International Publication Date: 18 April 1991 (18.04.91)
(21) International Application Number: PCT/US90/05682	(22) International Filing Date: 4 October 1990 (04.10.90)		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(30) Priority data: 417,357	5 October 1989 (05.10.89)	US	
(71)(72) Applicant and Inventor: KAWASAKI, Glenn [US/US]; 816 East Gwinn Place, Seattle, WA 98102 (US).			
(74) Agent: PARMELEE, Steven, W.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).			
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent).			

(54) Title: CELL-FREE SYNTHESIS AND ISOLATION OF NOVEL GENES AND POLYPEPTIDES**(57) Abstract**

A method for the cell-free synthesis and isolation of novel genes and polypeptides is provided. Within one embodiment, an expression unit is constructed onto which semi-random nucleotide sequences are attached. The semi-random nucleotide sequences are first transcribed to produce RNA, and then translated under conditions such that polysomes are produced. Polysomes which bind to a substance of interest are then isolated and disrupted; and the released mRNA is recovered. The mRNA is used to construct cDNA which is expressed to produce novel polypeptides.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CC	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark			US	United States of America

5

CELL-FREE SYNTHESIS AND ISOLATION OF
NOVEL GENES AND POLYPEPTIDES

Technical Field

The present invention generally relates to the synthesis and isolation of novel genes and polypeptides in vitro and, more specifically, to methods of generating and expressing semi-random DNA or RNA sequences, isolating novel genes from those sequences, and using those genes to create novel polypeptides.

15

BACKGROUND OF THE INVENTION

The isolation of novel genes and polypeptides from semi-random sequences is currently limited by the need to screen a large, genetically diverse population of cells in order to obtain the sequence(s) of interest. For example, a polypeptide string of 10 amino acids has 20^{10} or approximately 10^{13} possible permutations. If 10 of these permutations had a desirable characteristic (such as the ability to bind a specific antigen), then a population of 10^{12} would have to be screened for the expectation of finding one desirable novel gene. Through the use of conventional methods (expressing novel genes via microorganisms), the screening of a large number of new sequences for a specific property is virtually unfeasible, unless the novel gene provides the organism with a distinct growth or survival advantage. Indeed, under the current state of the art, the 10^{12} independently transformed microorganisms would have to be screened individually to locate that one desirable novel gene.

Within present screening procedures for detecting novel gene products which are localized within cells, colonies derived from each transformed cell must be treated to break open the cells. Typically 1000-2000 bacterial colonies per standard petri dish are lysed (e.g., by chloroform) for the screening procedure. Thus, to examine 10^{12} transformed

organisms, 500,000 to 1 billion petri dishes would be necessary. In addition, 10,000 to 100,000 liters of logarithmically dividing cells may be necessary for producing the large numbers of transformable cells.

5 Alternatively, where a gene product is secreted and attached to the outside of a cell, it may be detected by its ability to bind a fluorescent compound or other marker. In these cases, cell sorters may be used to screen for the synthesis of a novel desirable polypeptide. However, even at a
10 flow rate of 5,000 cells per second, it would take a cell sorter over 60 years to screen 10^{12} cells. Thus, present day screening methods which are both extremely costly and time-consuming, effectively prohibit the isolation of novel genes and polypeptide from semi-random sequences.

15 In addition to the methods briefly discussed above, Fields and Song (Nature 340:245-246, 1989) proposed a method for selectively obtaining polypeptides which specifically bind to other polypeptides, using the domains of the yeast GAL4 gene. However, this system has serious limitations. First,
20 only polypeptide-polypeptide binding may be selected; polypeptide-nonpolypeptide interactions are excluded. Second, both the known and novel binding polypeptides have to be expressed in yeast at reasonably high levels and in "native" conformations for the method to have commercial applicability.
25 Third, glycosylated polypeptides or polypeptides that have special modifications may also be excluded by this method. Fourth, it is not clear whether random or semi-random sequences can work, given that they used known polypeptides whose physical interactions were well-established and yet showed only
30 4.5% of the control GAL4 activity. Fifth, Fields and Song used very large sequences: 633 amino acids of the SNF1 protein and 322 amino acids of the SNF4 protein, which have evolved secondary structures that interact with each other. Sixth, using their method for semi-random sequences of even 10^{10}
35 diversity obviates the need for extremely large amounts of DNA, modifying enzymes, and competent yeast cells.

Contrary to previously disclosed methods, the present invention describes a method for cell-free screening of novel

genes and polypeptides. This method avoids the problems associated with large numbers of transformed organisms as well as the limitations of the method disclosed by Fields and Song, and may be completed within a few weeks. Therefore, the 5 methodology allows a substantial time and monetary saving in the isolation of novel gene products.

SUMMARY OF THE INVENTION

Briefly stated, the present invention relates to 10 methods for synthesizing, screening, and selecting high numbers of novel genes and polypeptides. The methods generally comprise the steps of (a) constructing an in vitro expression unit comprising a 5' untranslated region containing an RNA polymerase binding sequence, a ribosome binding sequence, and a 15 translation initiation signal, the expression unit being capable of producing mRNA; (b) attaching one or more semi-random nucleotide sequences to an expression unit; (c) transcribing or replicating the sequences associated with the expression unit and semi-random nucleotide sequences to produce 20 RNA; (d) translating the RNA to produce polysomes under conditions sufficient to maintain the polysomes; (e) binding the polysomes to a substance of interest; (f) isolating the polysomes that bind to the substance of interest; (g) disrupting the isolated polysomes to release mRNA; (h) 25 recovering and constructing cDNA from the released mRNA; and (i) expressing the gene to produce novel polypeptides.

In one embodiment of the method described above, the process may be repeated on mRNA that has been enriched for desirable sequences by amplifying the RNA or respective cDNA. 30 Subsequently, this amplified subset of genes may be cycled through the various steps outlined above to further enrich for desirable novel genes until desirable sequences represent a significant ($>10^{-3}$) fraction of the truncated population. In principle, the method may be repeated until the population of 35 genes is nearly homogeneous.

Within a second aspect of the present invention, a method for producing novel polypeptides is provided, comprising the steps of (a) constructing an in vitro expression unit

comprising a 5' untranslated region containing an RNA polymerase binding sequence, a ribosome binding sequence, and a translation initiation signal, the expression unit being capable of producing mRNA; (b) attaching one or more 5 semi-random nucleotide sequences to the expression unit; (c) transcribing sequences associated with the expression unit and semi-random nucleotide sequences to produce RNA; (d) translating the RNA to produce biologically active 10 polypeptides; (e) subdividing the RNA encoding the biologically active polypeptides; (f) transcribing, translating, and subdividing as set forth in steps (c)-(e) so that the gene of interest is isolated; (g) constructing cDNA from the isolated gene; and (h) expressing the cDNA to produce novel 15 polypeptides.

15 In yet another aspect of the present invention, a method of producing novel polypeptides is provided comprising the steps of (a) constructing an in vitro expression unit comprising a 5' untranslated region containing an RNA polymerase binding sequence, a ribosome binding sequence, and a 20 translation initiation signal, the expression unit being capable of producing mRNA; (b) attaching one or more semi-random nucleotide sequence to the expression unit; (c) replicating the sequences associated with the expression unit and semi-random sequences to produce RNA; (d) translating the 25 RNA to produce biologically active polypeptides; (e) subdividing the RNA encoding the biologically active polypeptides; (f) translating and subdividing as set forth in steps (d)-(e) such that the gene of interest is isolated; (g) constructing cDNA from the isolated gene, and (h) expressing 30 the cDNA to produce novel polypeptides.

The expression unit described above comprises an RNA polymerase binding sequence, a ribosome binding site, and a translation initiation signal. The expression unit may further comprise a translation enhancer or "activator" sequences, a 3' 35 tail of a selected sequence and appropriate restriction sites. The semi-random DNA sequences may be generated by mechanically, chemically, or enzymatically fragmenting naturally-occurring DNA, by chemically synthesizing the DNA, or by polymerizing the

DNA directly onto the expression unit. The substance of interest may be a surface antigen, receptor protein, toxin, organic polymer, active site of a protein molecule, metabolite, antibody, metal, hormone, or other compound.

5 These and other aspects will become evident upon reference to the following detailed description.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is directed to the isolation of 10 novel genes and polypeptides. These novel genes may have virtually infinite diversity and may code for new polypeptides with commercially important properties, such as novel catalytic activities or the ability to bind selectively to specific substances. Novel genes may be constructed which comprise open 15 reading frames from existing genes or from semi-random nucleotide sequences of chemically synthesized DNA. They may be expressed in a wide variety of organisms using existing promoters, enhancers, initiation codons, plasmids, ribosomal binding sites, and/or terminators. In some cases, it may be 20 advantageous to express the novel genes *in vitro*, as part of a large-scale production process.

As noted above, the present invention describes a multistep process for constructing and isolating novel genes and gene fragments which encode novel polypeptides with 25 specific binding and/or biological activities. Within a preferred embodiment, the process comprises the following steps:

1. An expression unit is constructed which contains an RNA polymerase binding sequence (i.e., a promoter or an 30 RNA-directed RNA polymerase initiation site), a ribosome binding site, and a translation initiation signal. The expression unit may also contain convenient restriction sites, translation enhancer or "activator" sequences, and a 3' tail of a selected sequence.

35 2. Semi-random DNA or RNA sequences are then generated by mechanically, chemically, or enzymatically fragmenting naturally-occurring DNA, RNA, or cDNA sequences, or by chemically synthesizing the nucleotides. The semi-random

DNA or RNA sequences are then inserted into the expression unit. Alternatively, the semi-random sequences can be polymerized directly onto the expression unit. A library of 10^{12} or greater different sequences may then be created.

5 3. The novel genes are then transcribed in vitro to produce a pool of RNA copies of the original DNA library. If an RNA-directed RNA polymerase sequence is included, then these replicases may be used to amplify the RNA.

10 4. The RNA (mRNA) is translated in vitro to produce polysomes. Conditions for maintaining the "polysomes" (RNA-ribosome-nascent polypeptide complexes) are used to keep the desired polypeptide and mRNA together.

15 5. The polysomes are then allowed to bind to substances of interest, such as surface antigens, receptor proteins, toxins, organic polymers, antibodies, metabolites, hormones, and active sites of protein molecules, or to display biological activity.

20 6. Polysomes binding to the substance(s) of interest are substantially enriched by the removal of the unbound polysomes. Serial or flow-through washes under conditions which maintain the polysome complexes substantially increase the frequencies of the desired mRNAs, which remain attached to the substances of interest through the polysome structure.

25 7. The bound/active polysomes are then disrupted to release the mRNAs from the polysome complex.

30 8. The rare mRNAs are then recovered by making cDNA copies or by direct amplification of the RNA with RNA-directed RNA polymerases. The amplification of the cDNA with DNA polymerase and/or reverse transcriptase reactions may allow greater ease in recovering these low abundance messages.

9. The resulting cDNAs are then expressed to produce polypeptides.

35 In most instances, repetition of steps 3-8 is preferable to further increase the frequency of specific binding proteins above a background of nonspecific binding of polysomes.

The isolated, purified novel gene(s) produced by the methods described herein are capable of generating a variety of polypeptide(s) of interest using standard expression techniques, as positive proof that the gene codes for the 5 desired product. In addition, DNA and/or polypeptide sequencing by conventional methods may be used to identify the composition of the novel polypeptide.

Once the polypeptide encoded by the novel gene has been isolated and identified, large-scale production of the 10 novel polypeptide(s) may be accomplished by chemical synthesis (if the amino acid sequence is relatively short) or through recombinant DNA methods, using genetically engineered microorganisms. Alternatively, large-scale *in vitro* transcription and/or translation methods may be used to produce 15 commercial quantities of the polypeptide.

The DNA sequence coding for the selected polypeptide may also be incorporated into larger genes (i.e., such as into the hypervariable regions of antibody genes) to create hybrid proteins with the specific binding and/or biological activities 20 of the originally isolated novel polypeptides, in addition to other binding and biological activities.

I. THE EXPRESSION UNIT

The expression unit comprises a 5' untranslated 25 region and may additionally comprise a 3' region. The 5' untranslated region of the expression unit contains a promoter or RNA polymerase binding sequence, a ribosome binding sequence, and a translation initiation signal. The 5' untranslated region ("head") may also contain convenient 30 restriction sites and a translation enhancer or "activator" sequence(s). The 3' region may contain convenient restriction sites and a 3' tail of a selected sequence. The expression unit may be chemically synthesized by protocols well known to those skilled in the art. Alternatively, these elements may be 35 incorporated into one or more plasmids, amplified in microorganisms, purified by standard procedures, and cut into appropriate fragments with restriction enzymes before assembly into the expression unit.

The 5' untranslated region contains a promoter or RNA polymerase binding sequence. High-efficiency promoters, such as those for the T7, T3, or SP6 RNA polymerase, are preferred in this invention for the following reasons. Such promoters 5 are short DNA sequences of known composition, are extremely specific for their relative polymerases, and are highly active, allowing for greater than 50 rounds of transcription per DNA template. In addition, T7, T3, and SP6 polymerases are commercially available from many sources and are components of 10 well-characterized transcription kits. For the T7 promoter, the consensus sequence is TAATACGACTCACTATAGGGAGA (23 base pairs). Although this sequence is described in conjunction with a preferred embodiment of this invention, it will be evident that related DNA sequences may be used which will 15 function for T7 RNA polymerase, and other sequences will be appropriate for other RNA polymerases. Within certain embodiments, it may be desirable to utilize two promoters, such as both the T7 and SP6 promoters.

Positioned downstream of or within the promoter 20 region is a DNA sequence which codes for a ribosomal binding site. This ribosome binding site may be specific for prokaryotic ribosomal complexes (including ribosomal RNAs) if a prokaryotic translation procedure is used. However, a preferred embodiment of this invention uses a eukaryotic 25 sequence and an in vitro eukaryotic translation system, such as the rabbit reticulocyte system (Krawetz et al., Can. J. Biochem. Cell. Biol. 61:274-286, 1983; Merrick, Meth. Enzymol. 101:38, 1983). A consensus translation initiation sequence, 30 GCCGCCACCATGG, as well as other functionally related sequences have been established for vertebrate mRNAs (Kozak, Nucleic Acids Res. 15:8125-8148, 1987). This sequence or related sequences may be used in the novel gene construction to direct 35 protein synthesis in vitro. The ATG triplet in this initiation sequence is the translation initiation codon for methionine; in vitro protein synthesis is expected to begin at this point.

Between the promoter and translation initiation site, it may be desirable to place other known sequences, such as translation enhancer or "activator" sequences. For example,

Jobling et al. (Nucleic Acids Res. 16:4483-4498, 1988) showed that the untranslated "leader sequences" from tobacco mosaic virus "stimulated translation significantly" in SP6-generated mRNAs. They also reported that the 36-nucleotide 5' untranslated region of alfalfa mosaic virus RNA 4 increases the translational efficiency of barley amylase and human interleukin mRNAs (Jobling and Gehrke, Nature 325:622-625, 1987). Black beetle virus (Nodavirus) RNA 2 (Friesen and Rueckert, J. Virol. 37:876-886, 1981), turnip mosaic virus, and 10 brome mosaic virus coat protein mRNAs (Zagorski et al., Biochimie 65:127-133, 1983) also translate at high efficiencies. In contrast, certain untranslated leaders severely reduce the expression of the SP6 RNAs (Jobling et al., ibid., 1988).

Appropriate restriction sites may also be included in the expression unit to assist in future genetic engineering. For example, the sextuplet, CCATGG, is the recognition sequence for the restriction endonuclease, NcoI. A NcoI "cutting site" positioned downstream of the ribosomal binding site is a 20 convenient splice point for subsequent genetic engineering. Hence, after purification of a desired novel gene, the expression unit may be spliced from the novel gene at this site, and another promoter may be attached for expression in vivo and large-scale production of the novel polypeptide. The 25 NcoI site may also be used as a convenient cloning site for the construction of hybrid proteins, where two different polypeptide domains are brought together and expressed as a single protein.

In addition, it is most likely advantageous to 30 include in the 5' untranslated region a DNA sequence with at least one restriction endonuclease site for subsequently cloning the novel gene into plasmids. The octameric sequence, GCGGCCGGC, is recognized by NotI nuclease and is particularly useful because it would rarely fall within the novel coding 35 region of the gene (NotI is expected to cut totally random DNA once every 65,536 base pairs). Other restriction sites may also be used; the expected frequency of cutting the novel coding region is dependent upon the nucleotide composition or

the DNA source of the coding region. It should be noted that certain palindromic sequences may interfere with translation; however, some sequences may also enhance the rate of translation.

5 The expression unit may also comprise a 3' region. It is desirable to construct known 3' regions (tails) with palindromic sequences for at least two reasons. First, 3' restriction sites would be convenient for any later genetic engineering of the polypeptide coding region. For example, if
10 NotI sites were located in both the 5' and 3' regions, a desirable polypeptide coding sequence could be cut out with NotI "sticky ends" for further cloning. Second, palindromes may cause secondary structures which impede translocation, thus, palindromes in the 3' region may slow down the movement
15 of ribosomes during translation. This second property may be desirable for preventing ribosomes from "falling off" the mRNA and thereby enhancing the number of polysomes in the in vitro translation step. The 3' region may also contain a poly-A or other polynucleotide stretch for later purification of the mRNA
20 from other components in the in vitro translation reaction by hybridization to a complementary homopolymeric sequence.

In addition, other nonrandom sequences may be incorporated into the expression unit. Within one embodiment, the expressed polypeptides contain both nonrandom and
25 semi-random amino acid sequences. The nonrandom component of the coding region is synthesized and produced with the nonrandom 5' untranslated region and/or with the 3' region. This nonrandom coding sequence specifies a string of amino acids (an identification or "ID" peptide) that is conserved
30 among the billions of novel polypeptides. The ID peptide would be useful for quantifying the amount of novel polypeptide and for purification of the novel polypeptide (given that an antibody against the ID peptide is available or can be produced). One example is the 11 amino acid Substance P, which
35 can be attached as a fusion peptide to other polypeptides. Anti-Substance P antibodies are commercially available for detecting and quantifying fusion proteins containing Substance

P. Another example is the eight amino acid marker peptide, "Flag" (Hopp et al., Bio/Technology 6:1204-1210, 1988).

Amino-terminal ID peptides have at least two advantages over carboxy-terminal ID peptides. First, it is easier to make gene constructions which maintain the proper reading frame of the N-terminal ID, because long stretches of semi-random DNA or RNA will tend to end in all three reading frames for a C-terminal ID. Second, the N-terminal ID may be designed to function as a signal peptide in a transformed organism, allowing for the possible secretion of the novel polypeptide during large-scale production.

Nevertheless, C-terminal ID polypeptides may also be used. One preferable C-terminal polypeptide is polyglycine, which is encoded by poly-dG and is read Gly-Gly-Gly, etc., regardless of the reading frame of the semi-random sequences. The polyglycine 3' end of the polypeptide may act as a noninterfering tether of the nascent peptide and allow the semi-random sequences greater access to bind molecules of interest. In addition, the poly-dG sequence may be used for priming second strand synthesis of the cDNA and may be useful for purification of the RNA or DNA with polyC or poly-dC. Other repetitive sequences, such as GGGCGGGC..., may be used to code for a recognizable peptide sequence which is expressed in all reading frames. A preferable form of the ID peptide is one which may be cleaved from the novel polypeptide by simple chemical or enzymatic means.

In addition to the DNA expression unit, an RNA expression unit may be constructed for semi-random polypeptide synthesis. One possible advantage of the RNA expression unit is that the recovery of the polysomal mRNA does not have to go through an initial cDNA stage. Instead, the mRNA with the desired sequences may be amplified with an RNA-directed RNA polymerase, such as that of QB (Q Beta) replicase (Haruna and Spiegelman, Proc. Nat. Acad. Sci. 54:579-587, 1965). This enzyme can make one billion copies of recombinant RNA in 30 minutes (Lizardi et al., Bio/Technology 6:1197-1202, 1988). One suitable cloning strategy for amplification of recombinant RNA is detailed in Lizardi et al. (ibid., 1988). For purposes

of the present invention, other elements, such as restriction sites, enhancers, and ID sequences, may be added to the DNA plasmids which give rise to the QB RNA templates. Semi-random coding sequences may be inserted on these plasmids by standard 5 DNA methodologies. When the QB replicase template is transcribed (for example, by T7 RNA polymerase), an RNA library capable of in vitro replication may be created which contains the semi-random gene sequences. Alternatively, a similar RNA expression unit may be constructed by chemically synthesizing 10 the appropriate RNA molecules and assembling them via an RNA ligase, such as the T4 RNA ligase (commercially available), which links together single-strand RNA and/or single-strand DNA.

15 II. SEMI-RANDOM NUCLEOTIDE SEQUENCES

Semi-random sequences of DNA or RNA are attached to the expression unit. Since the RNA expression units and semi-random sequences may be generated from a DNA template or constructed from chemically synthesized RNA or mRNA fragments 20 in much the same manner as DNA expression units, the following description merely describes the process for semi-random DNA attachment to the expression unit. Those skilled in the art will readily be able to construct the RNA-equivalent of the expression units attached to semi-random polynucleotides.

25 Semi-random DNA may be generated by at least three methods. First, naturally-occurring DNAs from virtually any living source may be mechanically, chemically, or enzymatically fragmented and attached to the 5' untranslated region with DNA ligase. Mixtures of fragments from different DNA sources may 30 be used. The end result may be the selectable expression of an active "open reading frame"--a portion (fragment) of a protein that has no "nonsense" (or "stop") codon, unless the activity resides in the extreme C-terminus of the molecule. In one embodiment of this invention, a gene coding for a known 35 function may be fragmented; the resulting pieces are ligated to the 5' untranslated region and later screened for the expression of activity in the polysome assay. By examining the smallest gene fragment which provides biological activity, an

analysis of protein domains may be made. Gene fragment analysis may be useful for creating small biologically active peptides and hybrid therapeutic proteins and may be beneficial for drug delivery, if smaller size assists the peptide in 5 reaching the target site.

In another embodiment of the present invention, the "fragmented" DNAs may be semi-randomly sized cDNA molecules from a cDNA library. By expressing cDNAs in vitro and using polysome selection, a very rare partial or perhaps even 10 full-sized gene may be isolated through binding the polysome to antibody, receptor protein, or other diagnostic molecule. The cell-free expression of cDNA "fragments" as herein described may be orders of magnitude more sensitive than previously described methods in locating desirable cDNA clones.

15 A second method for generating semi-random DNA is to chemically synthesize the DNA. For example, relatively long DNA molecules of approximately 100 nucleotides may be synthesized with mixtures of nucleotides at each position. However, a statistical problem of nonsense codons becomes 20 apparent with chemically synthesized DNA. For the gene fragments and cDNA strategies described above, an active, open reading frame is located from within existing protein sequences. "Open reading frame" implies that no stop codon exists and often indicates a sequence from within a protein 25 coding region.

However, it should be noted that chemically synthesized DNA having enough diversity to code for all 20 common amino acids at all positions may not necessarily have open reading frames. The stop codons--TAA, TAG, and 30 TGA--represent three of the 64 possible DNA triplets. For completely random DNA, with the equal likelihood of any of the four nucleotides in each position, the probability of a nonsense codon is therefore $3/64 = 4.6875\%$. For a random DNA stretch coding for a string of 30 amino acids, the probability 35 of at least one stop codon within that string is about 76%. Stop codons cause termination of translation and release of the nascent polypeptide from the ribosome complex. Therefore, strategies to reduce the frequencies of nonsense codons and to

bypass the usual result of nonsense codons during protein translation are preferable, and discussed below.

More specifically, the A, T, C, and G base composition may be manipulated to favor certain codons and in particular to reduce the likelihood of nonsense codons. In the extreme case, the third position of each triplet codon may be synthesized with only C and T to theoretically avoid nonsense codons. However, in this case not all 20 amino acids are encoded. Lim and Sauer (Nature 339:31-36, 1989) have used an equal mixture of all four bases in the first two codon positions and an equal mixture of C and G at the third codon position in synthesizing new regions of lambda repressor. This combination allows for any of all 20 amino acids at each codon and reduces the frequency of nonsense triplets to 1/32 = 3.125%. However, in a string of 30 amino acids the likelihood of at least one TAG stop codon is about 61%.

In a preferred embodiment of this invention, unequal mixtures of the bases are used in all three codon positions to reduce the frequency of stop codons, while still allowing a high frequency of all 20 amino acids at all codons. In the first codon position equal molar amounts of C, A, and G are used, but only half that amount of T is used. In the second codon position the amount of A is reduced to half of the level of the other three bases. In the third codon position only G and C or G and T are used, and in equal molar amounts. The result of this strategy is a greater than 79% probability that no stop codons will be present in a string of 30 amino acids. The proportions of the individual amino acids are slightly distorted in this case relative to a totally random DNA strategy. However, only tyrosine will be represented at less than half of the expected frequencies compared to the random situation.

To further overcome the presence of nonsense codons when using chemically synthesized DNA, it is preferred that nonsense suppressing tRNAs be used in the in vitro translation steps. In particular, since the strategy described above eliminates all but the TAG stop triplet, and tyrosine codons are underrepresented as the result of unequal mixtures of bases

at each codon position, a nonsense suppressor which recognizes TAG (actually UAG in the mRNA) and inserts tyrosine into the growing polypeptide chain is most desirable. Such tyrosine-inserting nonsense suppressors may be generated by 5 changing the anticodon region of a tyrosyl-tRNA in such a manner that the tyrosyl-tRNA now "reads" UAG instead of the normal UAU and UAC tyrosine codons in mRNA. Normal tyrosyl-tRNAs will also be included in the translation step to 10 read the tyrosine codons. Nonsense suppressors can also be made for the other two nonsense codons. As an example, 15 tryptophane- or leucine-inserting suppressors of the UGA stop codon have been well characterized--as have many other nonsense suppressors. The nucleotide sequences of many nonsense suppressors are known; and, therefore, the construction of such molecules would be evident to those skilled in the art.

Nonsense suppressors of mammalian translation systems are known (Burke and Mogg, Nucleic Acids Res. 13:1317-1326, 1985; Capone et al., EMBO J. 4:213-221, 1985; Diamond et al., Cell 25:497-506, 1981; Hudziak et al., Cell 31:137-146, 1982; 20 Laski et al., EMBO J. 3:2445-2452, 1984). Additionally, different investigators have shown that the "reading" of nonsense codons in eukaryotic in vitro translation systems is possible with the use of suppressor tRNAs, including the tyrosine-inserting UAG suppressor tRNA from yeast (Capecci et 25 al., Cell 6:269-277, 1975; Gesteland et al., Cell 7:381-390, 1976). Readthrough of the UAG stop codon by such yeast suppressors has been reported as high as 70% in vitro (Pelham, Nature 272:469-471, 1978). Geller and Rich (Nature 283:41-46, 1980) have successfully suppressed nonsense codons in 30 reticulocyte systems with yeast suppressor tRNAs and with bacterial suppressor tRNAs and tRNA synthetase. Therefore, the use of tRNA suppressors in the present invention to reduce premature release of polypeptides from the ribosomes during the translation step is well within the state of the art. 35 Furthermore, both Pelham (ibid., 1978) and Geller and Rich (ibid., 1980) describe high levels of naturally-occurring nonsense suppression in eukaryotic translation systems. In particular, Pelham shows that a particular UAG codon in tobacco

mosaic virus may be "read" (suppressed) nearly 40% of the time by "supraoptimal concentrations of Mg⁺²," or a reported 2.1 mM MgCl₂. This level of magnesium ion or higher may therefore be used advantageously within the present invention to increase 5 the readthrough of nonsense codons and to thereby reduce the problem of translation termination of longer semi-random nucleotide sequences.

In generating the semi-random DNA by chemical means, different mixtures of bases at selected codon positions may be 10 used to strongly bias in favor of or against a particular amino acid. For example, the elimination of G at position three in a codon prevents methionine and tryptophan from being included in the peptide. As another example, a nucleotide mixture which is biased toward a high-cysteine content may be desirable for 15 producing short peptides with internal disulfide bonds for structural rigidity. Such rigid peptides may bind other molecules more tightly.

Second-strand synthesis of these artificial nucleotide sequences may be accomplished by "random priming" 20 and extension with DNA polymerase and/or by including a poly-dX tail from which to prime with poly-dX'. Other methods, such as the use of terminal palindromes that create "hairpin loops" for self-priming, may be used for second strand synthesis. 100 µg of double-stranded DNA of 100 nucleotides contains about 10¹⁵ 25 molecules. If the semi-random synthesis strategy is used, the expectation is that each of these molecules codes for a different polypeptide. Therefore, a very large diversity in coding potential exists within laboratory bench-scale amounts 30 of DNA. Such a synthetic DNA molecule of 100 nucleotides is merely provided for purposes of illustration; longer sequences may also be synthesized. In addition, shorter synthetic molecules may be generated and ligated together to make semi-random sequences of any given length. Shorter molecules 35 are expected to preserve the reading frame of the synthetic DNA better than longer molecules, because each addition of chemically synthesized base is not 100%. Therefore, more nonsense codons may be avoided by the use of shorter artificial

DNA molecules. T4 RNA ligase or other means may be used to link together the short single-stranded DNAs.

A third method for generating semi-random DNA is to polymerize the molecules directly onto the 3' end of the 5' untranslated region. If no N-terminal ID sequence is used, the polymerization may occur immediately after the ATG initiation sequence or preferentially after the ATGG sequence--which preserves both the consensus vertebrate initiation site and the NcoI site. The most commonly used enzyme for this polymerization is terminal transferase (usually from calf thymus), which is routinely used for generating homopolymeric regions for DNA cloning. However, by mixing different deoxynucleotide triphosphates, semi-random heteropolymers of DNA may be synthesized on a DNA primer with a free 3'-OH. Again, the A, T, C, and G base composition may be manipulated to favor certain codons and reduce the frequencies of nonsense codon by controlling the relative concentrations of the four deoxynucleotide triphosphates. In particular, a lower amount of dATP should reduce the frequencies of nonsense codons (TAA, TAG, and TGA). *E. coli* DNA polymerase I is reported to carry out non-template (de novo) synthesis of DNA and may be used instead of terminal transferase (A. Kornberg, DNA Replication, W.H. Freeman & Co., San Francisco, Calif., 1980). Other enzymes or chemical methods may also polymerize DNA directly onto the expression units. Second-strand synthesis is most easily accomplished by random primer extension, but other methods may provide the same result. Again, the use of nonsense suppressing tRNAs may greatly assist in overcoming the problem of stop codons in this semi-random DNA sequence.

30

III. TRANSCRIPTION OF THE NOVEL GENES

If DNA expression units are used with the semi-random sequences, mRNA may be easily created with RNA polymerase. As discussed above, T7, T3, and SP6 RNA polymerases are commercially available and extremely active. As an example, a DNA expression unit with a T7 promoter is treated with T7 RNA polymerase according to manufacturers' specifications. Approximately 50 mRNA copies may be synthesized routinely for

each DNA molecule in 30 minutes. The DNA may be degraded with RNase-free DNase. If the original DNA library had a sequence diversity of 10^{12} molecules, the resulting mRNA pool should reflect the same level of diversity but now contain 50 or more 5 RNA copies of each different DNA molecule. An RNA library of 6 μ g may contain 50 copies of 10^{12} different mRNAs that are each capable of expressing a semi-random polypeptide of 30 amino acids. Since 6 μ g is easily manageable in small test tubes, standard laboratory tools and vessels may be used.

10 The 5' ends of mRNAs need to be modified with the addition of diguanosine triphosphate "caps" (or analogs) for efficient translation in eukaryotic systems. The 5' capped mRNA may be generated during in vitro transcription (Hope and Struhl, Cell 43:177-188, 1985) and/or in the in vitro 15 translation process (Krieg and Melton, Nucleic Acids Res. 12:7057-7070, 1984). To cap messages during transcription, an excess of diguanosine triphosphate or an analog thereof (m7G(5')ppp(5')G, from Boehringer Mannheim Biochemicals, for example) is used during the RNA polymerization relative to GTP. 20 An mRNA capping kit based on this method is commercially available from Stratagene (California), which claims that 90%-95% of the resulting RNA is capped.

25 If the expression unit is RNA-based, such as the QB replicase system, a few RNA copies may be generated with T7 or other promoter systems (see Lizardi et al., ibid, 1988) if the novel gene constructions involve a DNA plasmid. Once RNA copies exist (or if the novel genes were assembled at the RNA level), RNA-directed RNA polymerase is capable of making a virtually unlimited number of copies of the RNA library (one 30 billion copies are easily attainable). However, the diversity of the library remains the same. With RNA phages, such as QB, the library may be self-sustaining at the RNA level without the necessity of going through a DNA intermediate.

35 IV. TRANSLATION OF THE RNA

Several in vitro translation methods are widely known. For convenience, the rabbit reticulocyte or wheat germ systems may be used with minor modifications. In vitro

translation kits are available commercially. For example, the "Translation Kit, Reticulocyte, Type I" from Boehringer Mannheim Biochemicals has all components for 100 translation reactions. Each reaction has been optimized for approximately 5 1 μ g of mRNA in a 25 μ l volume. One μ g of mRNA is sufficient to code for over 4×10^{12} novel genes, as described above. Therefore, it is possible to translate extremely high numbers 10 of novel genes in relatively small volumes. For example, 10^{13} 80S ribosomes only weigh approximately 66 μ g. Because of the small size of the mRNA, only a few ribosomes per message are 15 expected to saturate the mRNAs.

As described in the protocol for the representative 20 translation kit noted above, GTP and m7G(5')ppp(5')G are required for the efficient translation of in vitro transcribed 25 RNA. Even if mRNA capping has been previously performed during transcription, as described above, it may be advantageous to add the diguanosine triphosphate (or analog thereof) and guanylyltransferase (Krieg and Melton, *ibid.*, 1984) to the 30 translation reaction. In the absence of capping during transcription, the two reagents are necessary for the efficient 35 translation of the mRNA. In particular, when QB constructions are translated, diguanosine triphosphate (or analog thereof) and guanylyltransferase may be necessary for capping the RNA molecules during translation.

Other techniques may also be employed to optimize 40 translation and especially ribosome attachment to the mRNAs. For instance, it may be desirable to add ribonuclease 45 inhibitors, such as heparin. Eukaryotic systems, such as the wheat germ and reticulocyte translation methods, may yield 50 similar results to prokaryotic systems. The prokaryotic systems have the advantages of smaller ribosomes and more readily available nonsense suppressor tRNAs. In addition, in 55 prokaryotic cells transcription and translation are often simultaneous reactions. In the absence of coupled 60 transcription and translation in prokaryotes, mRNA stability is greatly reduced. Therefore, a prokaryotic in vitro expression 65 system may be used which combines transcription and 70 translation.

As described above, a preferred embodiment of the present invention is the use of suppressor tRNAs (especially tyrosine-inserting suppressors), which may be produced through recombinant DNA technology and/or by the partial purification of these molecules from mutant cell lines. Radioactive amino acids, especially S35-methionine, may be useful for monitoring in vitro translation and for following low amounts of polysomes in subsequent steps.

After about 30-60 minutes, protein synthesis begins in the translation reactions. The precise time may be determined for any given set of translation conditions by the use of radioactive amino acids (such as S35-methionine) and monitoring TCA precipitable counts, which is indicative of polypeptide synthesis. After the onset of protein synthesis, cycloheximide at a final concentration of 1 μ g/ml is added to prevent the movement of the ribosomes on the mRNAs (Lynch, Meth. Enzym. 152:248-253, 1987). This level of cycloheximide and a Mg^{+2} concentration of 5 mM may be used to maintain the mRNA-80S ribosome-nascent polypeptide complexes (polysomes). Other ribosome inhibitors may also be used since cycloheximide, for example, will not work on prokaryotic ribosomes. However, in the absence of GTP the polypeptide release from the ribosomes should not normally occur.

25 V. BINDING POLYSOMES TO SUBSTANCES OF INTEREST

The list of potential compounds to which the nascent peptide might bind is virtually unlimited. The coupling chemistries to link these compounds to columns, matrices, filters, beads, etc., will depend to a great degree upon the nature of the compound. In some cases, whole cells or cellular fractions may be used to find peptides which bind to cellular components, such as receptor proteins and other membrane-bound molecules.

For many proteins and nucleic acids, binding to nitrocellulose or similar artificial surfaces is a property of the filters or fibres. In these cases, the substances of interest are "stuck" to the membranes by established protocols. Bovine serum albumin (BSA), gelatin, casein or nonfat milk, or

other proteinaceous material is then typically added in excess to bind up any "free" surface sites. For example, an antibody is first bound to nitrocellulose by placing a solution of the antibody on a nitrocellulose disk in a microtiter dish. After 5 absorbing the antibody to the nitrocellulose, the disk is washed by moving the nitrocellulose disk to fresh microtiter dishes containing saline. After the washes, the disk is placed in a microtiter dish containing gelatin in solution. The disk is then washed again with saline.

10 Before allowing the polysomes to bind substances of interest, it may be desirable to pre-absorb the polysome mix against BSA, gelatin, and in particular the proteinaceous material (blocking protein) used in excess as described above. In this manner, polysomes which bind to the blocking protein or 15 nonspecifically to any protein are removed. This pre-absorption step will lead to much greater specificity of polysomes binding to the substance of interest. For binding to specific antibodies (as in the case above), the pre-absorption step(s) may include another antibody, preferably of a similar 20 subclass, but having different variable/hypervariable regions. By screening out polysomes which bind generally to antibodies but not to the variable/hypervariable region, the present invention may be useful for selecting anti-idiotypic binding 25 proteins. Such molecules may have biological or enzymatic activity (as seen for some anti-idiotypic antibodies) or be useful as vaccines.

The binding of polysomes to substances of interest may be accomplished in the presence of $MgCl_2$ (5 mM) and RNase 30 inhibitors, such as heparin. In addition, specific incubation parameters--such as low or high temperature, high or low salt, or different pHs--may be used to locate polypeptides which bind conditionally, depending on the environment. Incubation times will depend upon the concentration of the bound substance of interest and upon the nature of such substance.

VI. ISOLATION OF POLYSOMES WHICH BIND TO SUBSTANCE(S) OF INTEREST

After allowing the polysomes to selectively bind to the substance(s) of interest, nonbinding polysomes are generally removed by washings. This wash should contain $MgCl_2$ and perhaps gelatin, BSA, or other proteins to help reduce nonspecific binding of polysomes. If radiolabeled amino acids are used in the translations, washes (serial or flow-through) should continue until little detectable change is observed in radioactive counts bound to the substance of interest. If the amino acids are not labelled, washes should continue until at least 10^{-6} dilution of the polysome solution is obtained.

Conditionally-binding novel peptides may be isolated after these washes by shifting the polysomes into the desired environment for nonbinding, such as higher temperature, different pH, high metal ion concentration, or low salt concentration. Those peptides (and their attached ribosome mRNA complexes) which do not bind under the second ("stringent") condition(s) will be released into the solution and represent potential conditionally-binding factors against the substance of interest. Once immobilized, conditionally-binding peptides may be used to purify substances of interest. Alternatively, conditionally-binding peptides may serve as reagents in monitoring environmental changes.

25

VII. DISRUPTION OF THE ISOLATED POLYSOMES

The isolated (bound) polysomes may be easily disrupted by the removal of Mg^{+2} (by dilution or via chelating agents) or through the destruction of proteins by a number of methods (proteases, chloroform, etc.). Although dilution is the easiest method, it may not result in as thorough a disruption of the polysomes as compared to other methods. The bound polysomes are placed in a solution lacking Mg^{+2} to liberate the mRNA; RNase inhibitors may be desirable.

35

Conditionally-binding polysomes, which were released under any of the desired environments, may be treated in a similar fashion to disrupt the polysomes and release their mRNAs.

VIII. RECOVERING MESSENGER RNA AND CONSTRUCTING cDNA

Theoretically, if a single polysome binding to the substance of interest carries a mRNA, its rare mRNA is capable of being isolated (recovered) from the entire library of mRNAs. 5 The mRNA may also be amplified by several techniques in order to facilitate isolation.

The use of the polymerase chain reaction (PCR) on a single copy of DNA and on rare mRNA is well documented. (For 10 review, see H.A. Erlich (ed.), PCR Technology, Stockton Press, New York, N.Y., 1989; M.A. Innis et al. (eds.), PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, Calif., 1989; H.A. Erlich (ed.), Polymerase Chain Reaction: Current Communications in Molecular Biology, Cold Spring Harbor 15 Press, Cold Spring Harbor, N.Y., 1989.) Briefly, the rare mRNA is first subjected to cDNA synthesis by standard means. Since the sequences of the 5' and 3' regions are known, specific primers may be used for cDNA synthesis. Second, the single cDNA may then be amplified through the use of specified primers 20 (even the same primers as those used in cDNA synthesis). The primers used for PCR may include sequences which restore the 5' and 3' regions of the original expression unit--that is, sequences which restore the promoter (e.g., the T7 polymerase 25 recognition sequence) and 3' region are desirable. By recreating the expression unit in this manner, repeated rounds of transcription-translation-polysome selection may be performed until virtually all of the selected genes code for 30 binding peptides. For expression units based on RNA phages, such as QB, recovery and amplification of the rare mRNA is simplified because each mRNA may be capable of replication to one billion copies or more, using the appropriate replicases.

IX. EXPRESSION OF NOVEL GENES

Once the novel genes have been isolated and 35 sequenced, they or related sequences may be (1) cloned, (2) chemically reproduced, (3) mutated, and (4) expressed by protocols well known in the art. Large-scale production of the novel polypeptide may be accomplished through recombinant DNA

methods, using genetically engineered microorganisms. A large variety of prokaryotic and eukaryotic expression systems exist for the in vivo synthesis of the novel binding peptide. The convenient NcoI site described above or other restriction sites 5 may be used to connect the coding region of the novel gene to a desired promoter. It will be evident to those skilled in the art that other gene splicing strategies exist as well. A translation stop codon and a transcription termination sequence may be added to the 3' end of the novel gene for proper 10 expression of the gene in a microorganism. This genetically engineered sequence may then be placed on a plasmid or vector and placed within a desired host cell by transformation, transduction, infection, electroporation, microinjection, or other similar methods. The novel peptide sequence may be 15 attached to a signal sequence for possible secretion from the microorganism and/or may contain ID peptides, as herein described, for quantifying and purifying the resulting gene product. The novel peptide or related sequence may be attached to other translated sequences to form a hybrid or fusion 20 protein which is similarly expressed in a genetically engineered organism. Alternatively, large-scale in vitro transcription and translation methods may be used to produce commercial quantities of the polypeptide.

Finally, if the amino acid sequence of the novel peptide is relatively short, currently available technologies 25 allow for the large-scale chemical synthesis of the polypeptide. Chemical synthesis of the novel peptide has advantages over the in vitro and in vivo expression systems. Among these advantages chemical synthesis (1) is a better 30 defined and therefore more reproducible system for synthesis, (2) has no contaminating sources of DNA and RNA, (3) has no contaminating sources of nucleases, proteases, and other modifying enzymes, and (4) provides a relatively pure product after synthesis.

35

X. REPETITIVE ENRICHMENT FOR SPECIFIC POLYSOMES

Depending upon the amount of background, nonspecific binding of polysomes to the substance(s) of interest, one may

choose to perform a few to many cycles of translation-transcription-binding-recovery as described above to increase the frequency of sequences which code for the desired polypeptide. For example, if each cycle increases the 5 frequency of the desired novel gene(s) by 10^4 , then three cycles may be sufficient for isolating a sequence which exists in the original library at a frequency of 10^{-12} . Each cycle may be completed in one to three days; and many steps of the process may be performed by automated workstations, or robots. 10 Therefore, many cycles may be routinely accomplished for a desired binding activity within one week.

XI. SCREENING FOR ACTIVITIES OF TRANSLATED PRODUCTS WITHOUT POLYSOME BINDING

15 One aspect of the present invention does not require polysome binding for gene isolation. Instead, in vitro translation is allowed to proceed to completion, with the resultant release of the new polypeptides from the ribosome. This is accomplished by the use of nonsense codons or by the 20 ribosomes "falling off" the end of the mRNAs. The new peptides may be separated from the ribosomes and other components of the translation reaction by gel filtration and/or centrifugation and/or other means, in order to concentrate the translation products. The peptide mixture is then challenged to exhibit 25 biological or enzymatic activity--for example, the peptides are assayed for mitogenic activity by treating tissue culture cells lacking a growth factor.

If biological or enzymatic activity is observed within the entire array or subset of the novel peptides, the 30 gene(s) which codes for this activity may be located by subdividing the original library or an RNA copy of the library and screening for activity in a subdivision. After successive subdivisions, the desired gene may be isolated to a pool containing (for example) less than 1,000 different sequences. 35 In theory, the desired gene may be completely isolated by subdivision (to a "pool" containing just that one gene). With PCR, QB replicase or other methods (as described above), the desired sequences may be amplified to a level where in vitro

transcription and translation produces a highly enriched peptide solution having the biological/enzymatic activity. At a frequency of 1 to 10^{-3} , the gene of interest may be readily isolated and cloned into appropriate expression systems, using 5 methods currently available.

XII. CELL-FREE IDENTIFICATION OF NOVEL GENES AND PEPTIDES

After a novel gene with putative binding or biological activity has been isolated, it may be demonstrated 10 that the purified sequence codes for the activity of interest by amplifying the DNA and/or RNA so that sufficient mRNA is produced for larger-scale in vitro translation. The 15 translation products of this purified sequence should be nearly homogeneous polypeptides having the assayable activity. The gene and/or the polypeptide may be sequenced by existing methods to establish the composition of the novel polypeptide. Alternatively, the purified gene may be cloned into 20 microorganisms for amplification and expression. Subsequently, biological/binding activities as well as sequence identity may be established for the novel gene and polypeptide.

XIII. CREATING NOVEL HYBRID PROTEINS

After the nucleic acid sequence has been determined for the novel gene, this sequence may be incorporated into 25 larger genes to create hybrid proteins, which have the characteristics of the novel peptide and other desirable properties. One class of hybrid proteins which may be created by this technology is characterized by specific binding to cells and cytotoxic abilities. For example, a cell surface 30 receptor-binding peptide may be joined to ricin or other toxins via DNA splicing methods. This type of hybrid protein may be used to selectively kill different cell types, including pathogens and tumor cells. The gene which encodes the hybrid 35 protein may be completely synthesized or result from splicing the appropriate gene fragments together. This gene may be expressed in a variety of expression systems.

A preferred embodiment of this invention is the replacement of variable and hypervariable regions of antibody

and antibody-like genes by novel gene sequences which code for binding activities against substances of interest. In this manner, a much greater range of diversity is possible against antigens of interest; and the screening process may be much 5 more efficient and time-saving than the production methods for monoclonal antibodies against the same antigens. These "custom" hybrid antibody genes may be expressed in a number of organisms to produce active antibodies with new specificities or properties.

10

XIV. OTHER COMMERCIAL USES OF THE INVENTION

The application of the present invention in diagnostic tests parallels the use of monoclonal/polyclonal antibodies, and is more advantageous, primarily because the 15 isolation of novel polypeptides as herein described may take considerably less time (one week versus a few months for antibodies). In addition, other advantages may be seen. The novel polypeptides may be considerably smaller molecules than the antibodies. Therefore, synthesis, purification, and/or 20 manufacturing of the novel peptides may be greatly simplified and cost-effective as compared to antibodies. The smaller size may also aid in stability, formulation, and in reaching the target molecules.

The novel polypeptides may be identifiable by (1) 25 fusing them to a biologically active peptide which has a quantifiable activity (such as peroxidase or other enzymatic activity), (2) synthesizing them with an ID peptide, described above, to which existing antibodies are known to bind, (3) radioactively labelling them, (4) chemically adding markers, 30 such as fluorescent dyes or metallic substances, or (5) any combination of the above. To increase specificity in the diagnostic use of the novel polypeptides, two or more different polypeptides may be used. In addition, novel polypeptides may be used as competitive binding elements in diagnostic tests 35 which rely upon competitive binding to antigens or substrates.

Another advantage of novel polypeptides generated via the present invention is that they may bind to many classes of molecules which would not elicit a strong immune response,

because some molecules are not complex enough or are too similar to an organism's resident compounds to trigger antibody formation. In addition, the use of novel polypeptides in diagnostic binding assays may have a much greater scope than 5 the traditional antibody-based methods.

The novel polypeptides of the present invention may also be used therapeutically as originally isolated or as part of fusion proteins. For example, if a novel polypeptide were selected to bind a given toxin, it might also neutralize the 10 toxin. If a new polypeptide is bound to a viral receptor site on a cell membrane or to the virus's attachment mechanism, infection of the cell may be diminished. As described earlier, 15 fusion proteins carrying novel polypeptide recognition sequences in addition to a toxin may be used to selectively kill diseased or malignant cells. The binding of novel sequences to infected or malignant cells may trigger an immune response against the cell-peptide complex and, therefore, may be useful in the control of disease.

20

EXAMPLES

The following examples are provided by way of illustration and not by way of limitation. Within the examples, standard reagents and buffers that are free from 25 contaminating activities (whenever practical) are used. It is preferred to exercise care to avoid ribonucleases and PCR product contamination.

30

EXAMPLE 1

SYNTHESIS OF A NOVEL GENE LIBRARY

The sequences and strategies for creating a novel gene library require careful planning by those skilled in the art. The 5' untranslated region of the expression unit contains an RNA polymerase site, a ribosome binding site, an initiation codon, and selected 5' untranslated sequences. The 35 polymerase binding site used in this example is the T7 promoter sequence: TAATACGACTCACTATAGGGAGA (23-mer), which is placed at the 5' end of the expression unit.

A rabbit reticulocyte system is used for translation of the RNAs synthesized from the T7 promoter. Therefore, the ribosome binding site should include at least part of the consensus sequence for eukaryotic untranslated regions. In her 5 review article, Kozak (ibid., 1987) suggests that very short untranslated regions (less than 10 nucleotides) do not initiate protein synthesis efficiently. A selected untranslated region of 36 nucleotides is used here. This untranslated region is derived from the naturally-occurring (36-base pair) upstream 10 sequence of the adult rabbit hemoglobin (alpha-globin):

ACACTTCTGGTCCAGTCCGACTGAGAAGGAACCACCATGG, where the underlined ATG represents the start of translation at a methionine initiation codon (Baralle, Nature 267:279-281, 1977). The rabbit alpha-globin untranslated sequence is chosen 15 because (1) it is expected to be a favorable substrate in a rabbit reticulocyte system and (2) it contains the important "motifs" of Kozak's model mRNA.

The alpha-globin sequence is modified in the following ways for in vitro gene expression. First, the 5' A 20 (underlined above) is replaced by a G, which may aid in the capping of the mRNAs (Green et al., Cell 32:681-694, 1983). Second, the G (underlined in the alpha-globin sequence) is replaced with an A to help eliminate a putative secondary 25 structure in the untranslated region of alpha-globin which is hypothesized to reduce the initiation of protein synthesis by 60% relative to the beta-globin mRNA (Baralle, ibid., 1977). This second change also creates a convenient GATC restriction site in the 5' untranslated region. The resulting leader sequence, including the ATGG of the coding region, is therefore 30 the following:

GCACATTCTGATCCAGTCCGACTGAGAAGGAACCACCATGG.

This leader sequence is placed immediately downstream from the T7 promoter.

The 3' region contains (1) a selected sequence for 35 specific-primer-directed DNA synthesis, (2) a GGG-rich region which codes for a polyglycine tether that gives the nascent polypeptide spatial freedom to bind the substance of interest, and (3) convenient restriction sites whose resulting RNA

secondary structure may impede the translocation of ribosomes off the mRNA. The polyglycine region comprises 20 codons for glycine; most of the glycine codons are adjacent GGG triplets, which code for glycines in all reading frames. However, some 5 of the glycine codons are GGT or GGA to keep the DNA strands in proper register. The restriction sites for Bam HI (GGATCC) and NotI (GCGGCCGC) are chosen to be placed very near the 3' end of the gene; in the mRNA these sequences are expected to form hairpin loops. To prevent second-strand self-priming (of 10 hairpin loops) by the NotI sequence, an addition of AAAA is made at the 3' end. The 3' region therefore has a general sequence of (GGG or GGT/A)₂₀ followed by GGATCCGGCGGCCGCAAAA. A specific sequence for this region is given below.

The semi-random gene sequence is synthesized with 15 known 5' and 3' ends which undergo basepairing and ligation with the fully described 5' untranslated region and 3' region segments. To achieve this end, the semi-random gene is synthesized with a 5' CACCATGG, which may basepair with the octamer CCATGGTG on the complementary strand of the 5' 20 untranslated region. The initiation (first) codon, ATG, is necessary for translation of the semi-random sequences. The subsequent G is the first position of the second codon and is constant to preserve the NcoI site at the front end of the gene. The rest of this second codon and the next 28 codons are 25 synthesized following the rules outlined earlier for reducing nonsense triplets. That is, in the first codon position, equal molar amounts of C, A, and G are used but only half that amount of T is used. In the second codon position, the amount of A is reduced to half of the level of the other three bases. In the 30 third codon position, only G and C are used, and in equal molar amounts.

After codon 30 is synthesized, GGTGGGGG is added. This sequence codes for two glycine residues and is used to 35 ligate the semi-random sequences to the 3' region, which has a complementary CCCCCACC overhang on the opposite strand. The result of this synthesis is a sequence that codes for virtually all 30 amino acid polypeptides (beginning with methionine) and has a polyglycine tether. The probability of no stop codons in

this string of triplets is approximately 80%. By using partially purified yeast tyrosine-inserting UAG suppressor tRNA (Pelham, *ibid.*, 1978) during the subsequent translation, over 90% of the semi-random sequences are expected to code for 5 full-length polypeptide.

The specific oligonucleotides to synthesize are listed below:

I. T7 Promoter & "Globin" Leader (for gene synthesis and PCR):

10 5' TAATACGACTCACTATAGGGAGAGCACTTCTGATCCAG
TCCGACTGAGAAGGAAC3'-OH

II. Anti-T7 Promoter & "Globin" Leader (for gene synthesis):

15 5' CCATGGTGGTTCTTCTCAGTCGGACTGGATCAGAAC
TCTCCCTATAGTGAGTCGTATTA3'-OH (5' kinased with T4 Polynucleotide Kinase)

III. Semi-Random Gene (for gene synthesis):

20 5' CACCATGG ... semi-random as described ...
GGTGGGGGG3'-OH (5' kinased with T4 Polynucleotide Kinase)

IV. Poly-Glycine & 3' Restriction Sites (for gene synthesis):

25 5' TGGGGGTGGTGGGGGGGGGGGGGGGAGGGAGGGGGGG
GGGGAGGGGGAGGTGGTGGATCCCGCGGCCGAAAA3'-OH (5' kinased with T4 Polynucleotide Kinase)

V. Anti-Poly-Glycine & 3' Sites (for gene synthesis):

30 5' TTTTGCAGCCGCGGGATCCACCACTCCCCCTCCCCCCCC
CCTCCTCCCCCCCCCCCCCACCACCCCCACCC3'-OH

VI. Anti-Poly-Glycine & 3' Sites (for cDNA synthesis and PCR):

5' TTTTGCAGCCGCGGGATCCACCACTCCCC3'-OH

35 Sequences I and II are mixed in equimolar amounts in standard TE Buffer and heated at 65°C for 5-10 min. The complementary sequences (which comprise the 5' untranslated region) are allowed to anneal at 50°-60°C for one hour or longer, are allowed to cool slowly to room temperature, and are

thereafter stored at 0°-4°C. Sequences IV and V are likewise treated to form the double-stranded 3' region. These duplexes each have an eight-base, single-stranded overhanging sequence which is complementary to the known ends of Sequence III.

5 Equimolar amounts of I/II duplex, IV/V duplex, and semi-random Sequence III are ligated with T4 DNA ligase overnight at 13°-15°C in Ligase Buffer. The ligation mix is then run on a 1.5% agarose gel to separate out the desired ligation product, which is approximately 200 base pairs (233 bp
10 if completely double-stranded, which it is not). The "200 bp" DNA band is gel purified with NA45 paper (S&S) or by any of several protocols. A total of 2.5 µg (representing approximately 10^{13} DNA molecules) or more is desirable.

15 Complete double-stranded synthesis of novel genes is accomplished with DNA Polymerase I, Klenow, using standard methods. The double-stranded 3' region provides a primer for the "second-strand" synthesis of the semi-random sequences. T4 DNA ligase is used to join the newly synthesized DNA to Sequence II, thereby filling the nick in the second strand.
20 The DNA library is phenol/chloroform extracted and ethanol precipitated.

25 10 µg of completely double-stranded DNA molecules has a sequence diversity of 4×10^{13} . This library may then be transcribed with T7 RNA Polymerase to yield translatable mRNAs. However, with each transcription, the DNA library is consumed, unless DNA copies are made. To replicate the DNA library, 100 ng aliquots are each distributed to 500-µl tubes for PCR amplification in 200-µl reactions. According to PCR
Technology, pp. 18-19 (Erlich, *ibid.*, 1989), each 200-µl PCR
30 reaction yields about 5.2 µg of DNA--or an approximately 50-fold duplication of DNA in each aliquot. The aliquots are pooled. The pooled sample contains on the average 50 copies of each semi-random sequence and therefore may be used repeatedly (50 times, for example) without a large loss of diversity for
35 each translation with T7 RNA Polymerase. If the library is to be replicated with PCR, then the Klenow filling and ligation steps, described above, may be unnecessary, since the Taq polymerase is capable of filling in the gap and

nick-translating DNA (D. H. Gelfand, PCR Workshop, Society of Industrial Microbiology Meeting, Seattle, Wash., 1989). After nick translation, the gene is double-stranded and able to be PCR amplified.

5 Examples of oligonucleotide primers for PCR amplification of the DNA library are listed above in sequences I and VI. Generally, oligonucleotides of 25-30 bases are used for PCR amplification; however, longer primers may be used. It is important that the primers do not share significant 10 homologies or complementary 3' ends. Sequences I and VI have noncomplementary ends and no obvious regions of extensive homology.

In addition, after translation of these novel gene sequences, the resulting mRNAs lack T7 promoter sequences. 15 Sequence VI is used as the primer for first-strand cDNA synthesis. Sequence I is used as the primer for second-strand synthesis and restores the T7 promoter to the cDNA. In this way, later rounds of translation are possible on the selected novel gene sequences. PCR amplification may be necessary if 20 the resulting cDNAs are relatively rare.

EXAMPLE 2

TRANSCRIPTION OF NOVEL GENES

The DNA library (or a representative aliquot of those 25 sequences) described in Example One is transcribed with T7 RNA polymerase. 2.5 μ g of this DNA codes for nearly 10^{13} different polypeptides. The DNA is capped during transcription with Stratagene's mCAP™ Kit, according to the manufacturer's specifications. Approximately 5-10 μ g of mRNA is expected. 30 Generally, with T7 RNA polymerase, nearly 10 times this level of RNA is synthesized; however, the conditions for the capping reaction limit mRNA production in this case. The DNA is removed with DNase I, provided in the kit. The capped mRNA is phenol/chloroform extracted and precipitated with ethanol. The 35 RNA is resuspended in 10 μ l of TE and stored at 0°-4°C.

EXAMPLE 3TRANSLATION OF NOVEL GENES

The capped mRNA is translated with Boehringer Mannheim Biochemical's rabbit reticulocyte kit, with all 20 amino acids at 312.5 $\mu\text{mol/l}$ each. Capped mRNA from Example 2 is added to each reaction at 0.5 μg per reaction and is treated according to the manufacturer's protocol. After around 60 minutes at 30°C, cycloheximide is added to a final concentration of 1 $\mu\text{g/ml}$. MgCl_2 is adjusted to 5 mM, and heparin is added to 0.2 mg/ml. The reactions are pooled and submitted to a discontinuous sucrose gradient, according to Lynch (ibid., 1987). The polysomes may be frozen at -70°C or used directly.

15

EXAMPLE 4IMMOBILIZATION OF ANTIBODIES
AS THE SUBSTANCE OF INTEREST

Antibodies may be used to select for novel binding peptides. Peptides which bind to the hypervariable/variable regions of the antibodies ("anti-id peptides") may behave like the original epitopes which were used as immunogens. Because the novel anti-id peptides may mimic the original epitopes, these peptides may be useful as vaccines and/or may demonstrate biological activities, in much the same way that anti-id antibodies have been shown to have biological (sometimes catalytic) activities.

Examples of useful antibodies are anti-fibronectin, anti-nerve growth factor, anti-CD4, and anti-tumor necrosis factor, which are all available from Boehringer Mannheim Biochemicals. In general, antibodies to receptor molecules, growth factors, surface antigens, and biologically active peptides, as well as neutralizing antibodies to toxins and diseases, are good candidates for which to isolate anti-id binding peptides that may have agonist or antagonist properties or serve as vaccines.

The antibodies are affixed to Immobilon™ PVDF (polyvinylidene difluoride) membrane from Millipore Corporation, according to Pluskal et al. (BioTechniques

4:272-283, 1986). For example, anti-fibronectin antibody (from clone 3E3, Boehringer Mannheim Biochemicals) is absorbed onto a 0.5 cm x 0.5 cm square of PVDF, that has been "wetted" with 100% methanol and washed twice with 0.9% (w/v) NaCl in 10 mM 5 Tris buffer pH 7.4 (Saline Buffer). The amount of antibody needed is dependent upon the binding parameters of the desired anti-id peptides(s); Immobilon™ PVDF is reported to bind 172 $\mu\text{g}/\text{cm}^2$ of IgG. For convenience, 1 μg of anti-fibronectin IgG₁ 10 in saline buffer is absorbed onto the PVDF square by incubating 15 at room temperature for at least two hours. The PVDF is then washed with the Saline Buffer twice. The membrane is next incubated with a "blocking solution," containing 5% (w/v) gelatin in saline buffer for at least two hours at room temperature, so that the gelatin is absorbed into unoccupied sites of the PVDF. The membrane is then washed twice with 0.1% 20 gelatin in saline buffer. A similar treatment is done with 10 μg anti-keratin antibody (from clone AE1, Boehringer Mannheim Biochemicals), which is the control IgG₁ as described below.

20

EXAMPLE 5POLYSOME BINDING TO ANTIBODIES

25 Polysomes with nascent semi-random peptides are incubated in 1-ml reactions, each containing PS Buffer (0.9% NaCl, 10 mM Tris pH 7.4, 1% gelatin, 15 mM MgCl₂, 0.2 mg/ml heparin, and 1 $\mu\text{g}/\text{ml}$ cycloheximide) and a PVDF square with 10 μg anti-keratin IgG₁, described in Example 4. This pre-absorption step is done at 0°-4°C with gentle agitation for four hours to select out nonspecific binding of polysomes to gelatin and IgG₁. The anti-keratin PVDF square is removed with 30 jewelers' forceps and is replaced with the anti-fibronectin PVDF square. The mixture is incubated for four more hours under the same conditions to allow specific polysome binding to the variable/hypervariable region of the anti-fibronectin antibody. The anti-fibronectin PVDF square is removed and 35 washed three times by transferring it serially to fresh PS buffer.

EXAMPLE 6RECOVERING NOVEL GENES WHICH CODE FOR
ANTI-ID PEPTIDES FROM POLYSOMES

5 The PVDF membrane, which holds the washed antibody-bound polysomes, is transferred to a tube containing 100 μ l of 0.1 mM EDTA and is gently shaken at room temperature for 5-10 minutes to disrupt the polysomes and liberate mRNA. The PVDF is removed, placed in a fresh tube of 0.1 mM EDTA, and 10 stored at 0°-4°C overnight or longer (as a back-up). The released mRNA from the first EDTA treatment is reverse transcribed; and the resulting cDNA is amplified, according to PCR Technology (ibid., 1989), p. 91, with slight modification. Instead of using random hexamer for priming the cDNA synthesis, 15 a sequence complementary with the known 3' region (such as Sequence VI listed earlier as the downstream primer) is used for both cDNA synthesis and PCR reactions. The reverse transcriptase step is done in 100 μ l of PCR buffer with the appropriate relative amounts of the other reagents (instead of 20 μ l reaction). After the reverse transcriptase reaction, the mixture is split into 20 μ l aliquots; and each aliquot is amplified as described in PCR Technology, using Sequence I or a similar DNA upstream primer. After PCR amplification, the five 25 aliquots are pooled, phenol/chloroform extracted, and ethanol precipitated. This cDNA is then resuspended in TE and stored at 0°-4°C.

 The selected DNA is transcribed with T7 RNA polymerase and translated in a reticulocyte system, as previously described. In this case, the desired sequences are 30 greatly amplified compared to the original DNA library. By repetition of this cycle, which is greatly aided through the use of programmable workstations, desirable novel genes are concentrated to a level where conventional cloning and expression methods are practical. In addition, by dilution to 35 low Poisson Distribution of genes, a single novel gene(s) may be isolated, amplified, transcribed, and translated to demonstrate specific binding capability of the gene product(s).

Once binding has been demonstrated, the isolated gene(s) and polypeptide(s) may be sequenced for identification.

After the sequence of the novel binding peptide is known, many methods exist for the manipulation and large-scale synthesis of the peptide, as described herein.

EXAMPLE 7
COMPETITION ASSAY FOR BINDING PEPTIDES

10 After novel genes which code for binding peptides are selected, the amplified pools of recovered cDNA are assayed for the presence of the genes. Where ID sequences have been intentionally included to be coexpressed with the semi-random DNA sequences, ELISAs or other immunological assays for the 15 known part of the peptide are used to detect the binding of the novel portion of the peptide to the substance of interest. However, when no ID sequence is present and/or a confirmation of binding specificity is desirable, competition assays for the peptides are carried out. Competition assays, including 20 competition ELISA tests, are used to monitor for the presence of binding sequences within the various cDNA pools generated by the present invention.

One example is the screening of the cDNA pools for genes which encode peptides which bind anti-Pseudomonas 25 exotoxin (anti-PE) antibody. After two rounds of selection for polysome binding to the anti-PE antibody, different aliquots of the resultant cDNA pool were each transcribed in a 200- μ l reaction with T7 RNA polymerase (30 units) under standard conditions, starting with approximately 200 ng of DNA. The 30 mRNA products were phenol/chloroform/isoamyl alcohol extracted and precipitated with sodium acetate and ethanol at -20°. The precipitates were each centrifuged and resuspended in 16 μ l of distilled water which had been treated with diethylpyrocarbonate to remove nucleases.

35 The resuspended mRNAs were heated to 65° for five minutes and then placed on ice. The RNAs were translated with a wheat germ kit (Boehringer Mannheim Biochemicals) according to the manufacturer's recommendations. Each RNA sample was

expressed in a 50 μ l translation reaction with 25 microcuries of 35S-methionine and 0.5 μ l of RNase inhibitor. The reactions were run for 15-60 minutes at 30°. At the end of the translation, the samples were each equally divided: one half 5 was used to bind the substance of interest without competing substrate, while the other half was used to bind the substance of interest in the presence of excess competing substrate. In this case, the substance of interest was anti-PE antibody. The competing substrate was a 14-amino acid peptide (PE peptide), 10 which is derived from the toxin protein sequence and known to bind the antibody. The PE peptide sequence is Val-Glu-Arg-Leu-Leu-Gln-Ala-His-Arg-Gln-Leu-Glu-Glu-Arg. See Wozniak, et al., Proc. Natl. Acad. Sci., 85: 8880-8884 (1988).

The competition assays were done over ice in 96-well 15 microtiter dishes with flat bottoms. Immobilon PVDF disks were made with a standard 1/4 inch holepunch and placed in wells labelled "A". 50 μ l of methanol were added to the disks in "A" to wet and sterilize the membranes. The disks were transferred with forceps to wells "B" which contained 200 μ l of Saline 20 Buffer plus 10 mM MgCl₂ (TSM buffer). The disks were further washed by moving them to wells "C" which also contained 200 μ l of TSM. They were then transferred to wells "D" which contained 25 μ l TSM plus 3 μ l of anti-PE antibody (4.6 μ g/ μ l). The antibody was absorbed to the disks for three hours on ice 25 with gentle rotation (50-100 RPM on a platform shaker). Afterwards, 75 μ l of 2% nuclease-free BSA was added to "D" and absorbed for 1 hr. at 100 RPM.

The disks were washed twice in 200 μ l of TSM plus 0.1% BSA (in wells "E" and "F") for 30 minutes in each well and 30 were then ready for peptide binding. In wells "G" 26 μ l of TSM plus 0.1% BSA was mixed with 25 μ l of each translation reaction described above--half of the 50 μ l wheat germ system. Into one-half of each of the "G" wells, 1 μ l of PE peptide (1 mg/ml in TSM) was added to competitively inhibit the binding of novel 35 radioactively-labelled peptides to the antibody; these wells were labelled "+ Peptide." Into the control "G" wells, 1 μ l of TSM was added and the wells labeled: "No Peptide." The disks were added to the appropriate "G" wells and incubated to three

hours at 100 RPM on ice for peptide binding to the immobilized antibody.

After the binding reaction each disk was serially washed eight times in 200 μ l of fresh TSM at 0°, with a 10 minute incubation for each wash. The bound radioactivity for each disk was measured in a liquid scintillation counter with a 1 ml cocktail of Ecoscint. The following table lists the results of competition assays on different aliquots of cDNAs obtained from the binding of polysomes to the anti-PE antibody:

10

	<u>SAMPLE</u>	<u>CPM 35S-MET</u>
	WE1 + Peptide	6969
	WE1, No Peptide	8337
15	WE2 + Peptide	6163
	WE2, No Peptide	7693
	WP1 + Peptide	5792
20	WP1, No Peptide	6303
	WP2 + Peptide	5845
	WP2, No Peptide	6398

25 In each case the competing PE peptide reduced the amount of binding of the radioactively-labelled translation products of the selected cDNA pools, compared to the No Peptide controls. These results indicate the presence of gene sequences which code for binding peptides to the anti-PE antibody. Isolation and characterization of these DNA sequences is then done by cloning individual genes into plasmids, such as pUC18, pUC19, Bluescript, and many other available vectors.

30 From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for the purposes of illustration, various modifications may be made without deviations from the spirit

and scope of the invention. Accordingly, the invention is not to be limited except as by the following claims.

WHAT IS CLAIMED IS:

1. A method for producing novel polypeptides, comprising:

5 (a) constructing an in vitro expression unit comprising a 5' untranslated region containing an RNA polymerase binding sequence, a ribosome binding sequence, and a translation initiation signal, said expression unit being capable of producing mRNA;

10 (b) attaching one or more semi-random nucleotide sequences to said expression unit;

(c) transcribing or replicating the sequences associated with the expression unit and semi-random nucleotide sequences to produce RNA;

15 (d) translating said RNA to produce polysomes under conditions sufficient to maintain said polysomes;

(e) binding said polysomes to a substance of interest;

(f) isolating said polysomes that bind to said 20 substance of interest;

(g) disrupting said isolated polysomes to release mRNA;

(h) recovering said mRNA;

(i) constructing cDNA from said recovered mRNA; and

25 (j) expressing said cDNA to produce novel polypeptides.

2. The method of claim 1 wherein, subsequent to the step of recovering mRNA and constructing cDNA, amplifying said 30 cDNA by polymerase chain reaction.

3. The method of claim 1 wherein said semi-random nucleotide sequence comprises deoxyribonucleic acid.

35 4. The method of claim 1 wherein said semi-random nucleotide sequence comprises ribonucleic acid.

5. The method of claim 1 wherein said expression unit includes at least one RNA-directed RNA polymerase recognition sequence.

5 6. The method of claim 5 wherein said RNA-directed RNA polymerase is Q-Beta replicase.

10 7. The method of claim 1 wherein, subsequent to the step of recovering, amplifying the mRNA.

15 8. The method of claim 7 wherein the step of amplifying comprises synthesizing duplicate sequences with an RNA-dependent RNA polymerase.

15 9. The method of claim 8 wherein the RNA-dependent RNA polymerase is Q-Beta replicase.

20 10. The method of claim 1 wherein the step of isolating comprises removing polysomes that do not bind to said substance of interest by serial dilution or flow-through wash steps.

25 11. The method of claim 1 wherein, subsequent to the step of isolating said polysomes, said polysomes are exposed to selected stringency conditions such that said polysomes are released from said substance of interest.

30 12. The method of claim 11 wherein the step of exposing said polysomes comprises raising the temperature, lowering the salt concentration, or raising the metal ion concentration of said polysomes.

13. A method for producing novel polypeptides, comprising:

35 (a) constructing an in vitro expression unit comprising a 5' untranslated region containing an RNA polymerase binding sequence, a ribosome binding sequence, and a

translation initiation signal, said expression unit being capable of producing mRNA;

(b) attaching one or more semi-random nucleotide sequences to the expression unit;

5 (c) transcribing the sequences associated with the expression unit and semi-random nucleotide sequences to produce RNA;

(d) translating said RNA to produce biologically active polypeptides;

10 (e) subdividing the RNA encoding said biologically active polypeptides;

(f) transcribing, translating, and subdividing as set forth in steps (c)-(e), such that the gene of interest is isolated;

15 (g) constructing cDNA from said isolated gene; and

(h) expressing said cDNA to produce novel polypeptides.

14. A method for producing novel polypeptides, 20 comprising:

(a) constructing an in vitro expression unit comprising a 5' untranslated region containing an RNA polymerase binding sequence, a ribosome binding sequence, and a translation initiation signal, said expression unit being capable of producing mRNA;

(b) attaching one or more semi-random nucleotide sequences to the expression unit;

25 (c) replicating the sequences associated with the expression unit and semi-random nucleotide sequences to produce RNA;

(d) translating said RNA to produce biologically active polypeptides;

(e) subdividing the RNA encoding said biologically active polypeptides;

30 (f) translating and subdividing as set forth in steps (d)-(e) such that the gene of interest is isolated;

(g) constructing cDNA from said isolated gene; and

(h) expressing said cDNA to produce novel polypeptides.

15. The method of claim 14 wherein, subsequent to
5 the step of subdividing the RNA, amplifying the novel gene sequences associated with the biologically active polypeptides with polymerase chain reaction or with an RNA-directed RNA polymerase.

10 16. The polypeptide produced by the method of claim 1, 13 or 14.

15 17. The method of claim 1, 13 or 14 wherein said ribosome binding site comprises eukaryotic, prokaryotic, or viral ribosome binding sequences.

20 18. The method of claim 1, 13 or 14 wherein said ribosome binding sequence comprises the vertebrate consensus translation initiation sequence, GCCGCCACCATGG, or functionally related sequences.

25 19. The method of claim 1, 13 or 14 wherein the expression unit further comprises a sequence which codes for a selected amino-terminal ID peptide, said sequence positioned at the initiation codon.

30 20. The method of claim 1, 13 or 14 wherein said expression unit further comprises a 3' region of a selected sequence, said selected sequence selected from the group consisting of sequences enhancing the amplification, cloning, replication, purification, and isolation of the novel genes.

35 21. The method of claim 20 wherein said 3' region includes palindromic sequences which are adapted to impede ribosome translocation.

22. The method of claim 20 wherein said 3' region includes a C-terminal ID sequence.

23. The method of claim 22 wherein said C-terminal ID sequence comprises a repetitive sequence.

5 24. The method of claim 22 wherein said C-terminal ID sequence codes for a peptide capable of binding to antibodies.

10 25. The method of claim 1, 13 or 14 wherein said expression unit further comprises restriction sites adapted to allow expression of the novel gene in vivo.

15 26. The method of claim 25 wherein at least one of said restriction sites comprises the sequence CCATGG, said sequence positioned at the start of translation.

27. The method of claim 1, 13 or 14 wherein said expression unit includes the promoter sequences for T7, T3, or SP6 polymerase.

20 28. The method of claim 1, 13 or 14 wherein the semi-random nucleotide sequences are generated by mechanically, chemically, or enzymatically fragmenting naturally-occurring DNA or cDNA.

25 29. The method of claim 1, 13 or 14 wherein the semi-random nucleotide sequences are generated by chemically synthesizing nucleotides to form gene sequences.

30 30. The method of claim 29 wherein the step of synthetically synthesizing said nucleotides comprises the steps of (1) utilizing substantially equal molar amounts of C, A, and G, and only half of said substantially equal molar amount of T in the first codon positions; (2) utilizing substantially equal molar amounts of C, T, and G, and only half of said substantially equal molar amount of A in the second codon positions; and (3) utilizing substantially equal molar amounts of C and G or T and G in the third codon positions.

31. The method of claim 1, 13 or 14 wherein the step of attaching further comprises polymerizing said nucleotides directly onto the 3' end of the 5' untranslated region of the expression unit.

32. The method of claim 1 or 13 wherein the step of transcribing comprises transcribing said sequence in the presence of diguanosine triphosphate or analogs thereof.

10

33. The method of claim 1, 13 or 14 wherein the step of translating comprises translating said sequences in the presence of diguanosine triphosphate or analogs thereof and guanylyltransferase.

15

34. The method of claim 1, 13 or 14 wherein the step of translating is conducted in the presence of nonsense-suppressing tRNAs.

20

35. The method of claim 34 wherein the nonsense-suppressing tRNA is a tyrosine-inserting, nonsense-suppressing tRNA.

25

36. The method of claim 1, 13 or 14 wherein said substance of interest is selected from a group consisting of surface antigens, receptor proteins, toxins, organic polymers, metabolites, active sites of protein molecules, hormones, antibodies, and pollutants.

30

37. The method of claim 1, 13 or 14 wherein said substance of interest is the variable/hypervariable region of an antibody.

35

38. The method of claim 1, 13 or 14 wherein said substance of interest is a receptor protein.

39. The method of claim 38 wherein said receptor protein is a growth factor receptor protein.

40. The method of claim 39 wherein said growth factor receptor protein is selected from the group consisting of insulin and epidermal growth factor.

5

41. The method of claim 1, 13 or 14 wherein said substance of interest is selected from the group consisting of viral surface antigen, viral receptor protein and CD4.

10

42. The method of claim 1, 13 or 14 wherein the step of expressing cDNA comprises chemically synthesizing the amino acid sequence based on the nucleotide sequence of said cDNA.

15

43. The method of claim 1, 13 or 14 wherein the step of expressing cDNA comprises cloning the nucleotide sequence into an expression vector for synthesis in genetically engineered microorganisms.

20

44. The method of claim 1, 13 or 14 wherein the step of expressing cDNA comprises in vitro transcription and/or translation of the nucleotide sequence.

25

45. The method of claim 1, 13 or 14 wherein the step of expressing cDNA comprises synthesizing a nucleotide sequence encoding a polypeptide substantially homologous to that encoded by said cDNA, the polypeptide encoded by said nucleotide sequence being substantially identical to the binding region of said polysomes that bind to the substance of interest.

30

46. The method of claim 1, 13 or 14 wherein said cDNA is joined to other selected nucleotide sequences selected from the group consisting of sequences encoding toxins, antibodies, enzymes, biologically active peptides, and peptides capable of binding to antibodies.

35

47. A method for isolating a nucleotide sequence which encodes a polypeptide of interest, comprising

transcribing an in vitro expression unit which comprises a 5' untranslated region containing an RNA polymerase binding sequence, a ribosome binding sequence, a translation initiation signal, and one or more semi-random nucleotide sequences to produce a mRNA library;

5 translating the mRNA library under conditions which maintain polysomes having polypeptide chains attached thereto;

contacting the polysomes to a substance of interest and isolating mRNA from polysomes that specifically bind to the 10 substance of interest.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/05682

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 21/00; C12P 21/02; C12P 19/34;

U.S. CL: 435/69.1; 435/69.7; 435/91; 435/172.1; See Attachment

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S.	435/69.1; 435/69.7; 435/91; 435/172.1; 435/172.3; 536/27; 530/350

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

DATABASES: Dialog Service Online (Files, 1967-1990; Automated Patent System (File JPO and USPAT, 1975-1990)

Keywords: polysome, cDNA, immuno?, in vitro translation

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁴
Y	UK, A, 2,183,661 (BALLIVET ET AL) 10 JUNE 1987, see pages 1-10.	1,3,4,10-14, 16,17,19,20, 22,24-26, 28-47
Y	METHODS IN ENZYMOLOGY. Vol. 152, issued 1987. Lynch, "Use of Antibodies to Obtain Specific Polysomes," pages 248-252, see entire document.	1,3,4,10-14, 16,17,19,20, 22,24-26, 28-47
Y	CANADIAN JOURNAL OF BIOCHEMISTRY AND CELL BIOLOGY, Vol. 61, issued 1983, Krawetz et al., "In vitro Translation of Elastin mRNA and Processing of the Translated Products and the Signal Sequence Of Elastin a," pages 274-286, see entire document.	1,3,4,10-14, 16,17,19,20, 22,24-26, 28-47

See Attachment

- Special categories of cited documents: 15
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ⁸:

18 December 1990

Date of Mailing of this International Search Report ⁹:

04 FEB 1991

International Searching Authority ¹⁰:

ISA/US

Signature of Authorized Officer ¹¹

Jerome W. Marzil, Jr.
Richard Lebovitz

PCT/US90/05682

Attachment To PCT/IPEA/210
Classification Of Subject Matter

IPC(5): C12N 15/00; C12N 15/10; C07K 7/00; C07H 15/12
U.S. CL: 435/172.3; 536/27; 530/350

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	NUCLEIC ACID RESEARCH, Vol. 15, No. 20, issued 1987, Kozak, "An Analysis of 5'-noncoding Sequences From 699 Vertebrate Messenger RNAs," pages 8125-8148, see entire	18
Y	BIOTECHNOLOGY, Vol. 6, issued October 1988, Lizardi et al., "Exponential Amplification of Recombinant-RNA Hybridization Probes," pages 1197-1202, see entire document.	5-9,15
Y	US, A, 4.710.464 (BELAGAJE ET AL) 01 DECEMBER 1987, see e.g., ABSTRACT.	21,23

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter¹ not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.
 No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	NUCLEIC ACID RESEARCH, Vol. 12, No. 18, issued 1984, Krieg et al., "Functional Messenger RNAs are Produced by SP6 <u>in vitro</u> Transcription of Cloned cDNAs," pages 7057-7070, see especially Figure 6 on page 7067.	1, 3, 4, 10-14, 16, 17, 19, 20, 22, 24-26, 28-47
Y	US. A, 4,683,195 (MULLIS ET AL) 28 JULY 1987, see entire document.	2, 27